Interactions of lactic acid bacteria and yeasts originating from sourdough

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Tiivistelmä – Referat – Abstract
Sourdoughs are a natural habitat for microbial communities predominated by lactic acid bacteria (LAB) and yeasts. How microbial communities assemble and function is, to a large extent, determined by inter-species interactions. However, evidence for LAB-yeast interactions in rich environments, such as sourdough, is yet largely unavailable.

In this study, a set of LAB and yeast species was isolated from rye and wheat sourdoughs. While mainly typical sourdough species were identified, *Pediococcus parvulus* was, to the best of our knowledge, for the first time isolated from sourdoughs. The isolates were characterized in rich chemically defined culture conditions and screened for interactions. Potential interactions were discovered where LAB growth was enabled by a yeast, or where stable communities were formed despite competition. These findings, the resource of naturally co-occurring species, and the designed chemically defined growth medium present the grounds for future research for uncovering the underlying mechanisms of LAB-yeast interactions in rich environments.

LAB and yeasts commonly co-occur rich environments of fermented food processes and also in human gut and soil microbiomes. Therefore, the outcomes of this study support not only the optimization of food fermentations but provide also model systems for complex communities directly influencing human health.

Avainsanat – Nyckelord – Keywords
Lactic acid bacteria, yeasts, sourdough, fermented foods, microbial communities, microbial interactions

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Tiivistelmä – Referat – Abstract
Hapanjuuret ovat luonnollisia elinympäristöjä maitohappobakteerien ja hiivojen hallitsemille mikrobiyhteisöille. Mikrobiyhteisöjen muodostuminen ja toiminta riippuvat lajien välisistä vuorovaikutuksista. Näyttöä maitohappobakteerien ja hiivojen välisistä vuorovaikutuksista rikkaissa kasvuympäristöissä, kuten hapanjuurissa, on kuitenkin toistaiseksi hyvin vähän.

Tässä tutkimuksessa ruis- ja vehnähapanjuurista eristettiin maitohappobakteeri- ja hiivalajeja. Lajit olivat pääasiassa tyypillisiä hapanjuurilajeja, mutta myös *Pediococcus parvulus* eristettiin parhaan tietomme mukaan ensimmäisen kerran hapanjuuresta. Eristetyt lajit karakterisoitiin rikkaissa määritellyissä kasvatusoloissa ja niitä seulottiin vuorovaikutusten osalta. Tutkimuksessa havaittiin vuorovaikutuksia, joissa hiiva mahdollisti maitohappobakteerien kasvun, ja stabiileja yhteisöjä havaittiin muodostuvan kilpailusta huolimatta. Nämä seulotut löydökset, luonnollisesti yhdessä esiintyvien lajien kokoelma, ja kehitetty kemiallisesti määritelty kasvatusalusta mahdollistavat maitohappobakteerien ja hiivojen rikkaassa ympäristössä tapahtuvien vuorovaikutusten mekanismien selvittämisen.

Maitohappobakteerit ja hiivat esiintyvät tavallisesti yhdessä rikkaissa ympäristöissä, kuten ruokien fermentointiprosessissa, mutta myös ihmisen suolistossa ja maaperän mikrobiomissa. Näin ollen, tämän tutkimuksen tulokset tukevat fermentointiprosessien optimoinnin lisäksi myös monimutkaisempien mikrobiyhteisöjen, kuten ihmisen suolistomikrobiomin ymmärtämistä.

Avainsanat – Nyckelord – Keywords
Maitohappobakteerit, hiivat, hapanjuuri, fermentoidut ruoat, mikrobiyhteisöt, mikrobi-interaktiot

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Introduction

Lactic acid bacteria (LAB) and yeasts co-occur in a plethora of microbial communities. These include microbiomes in environments like soil (Chen *et al.*, 2005; Yurkov, 2018) and human gut (Lozupone *et al.*, 2012) or processes such as composting (Partanen *et al.*, 2010) and food fermentations (Wolfe and Dutton, 2015; Sieuwerts *et al.*, 2018). In food fermentation processes, LAB produce lactic acid acidifying the environment, antimicrobial compounds that protect the food matrix from contaminants, and aromatic compounds that contribute to flavor formation (Erten *et al.*, 2014). Yeasts in turn ferment sugars producing ethanol, carbon dioxide (CO_2) and secondary metabolites affecting the flavor and texture of end-products. Co-fermentation by LAB and yeasts is involved in a wide range of fermented foods, such as alcoholic beverages (Faria-Oliveira *et al.*, 2015), cocoa beans (Ho *et al.*, 2015), dairy products (Narvhus and Gadaga, 2003), vegetables (Swain *et al.*, 2014) and sourdoughs (De Vuyst *et al.*, 2014). When compared to non-fermented foods, fermented food products are found to have extended shelf-life, enhanced nutritional value, increased digestibility of food raw materials, and improved microbial stability and safety (Smid and Lacroix, 2013).

Sourdough is a mixture of water and cereal flour fermented by a community of microbial species, consisting mainly of LAB and yeasts (Gobbetti, 1998; Van Kerrebroeck *et al.*, 2017). Sourdough is used to make various end-products, but most traditionally sourdough bread. From perspective of the microbes, sourdough environment is relatively selective with its low pH and oxygen limitation (De Vuyst *et al.*, 2014). This is a characteristic environment also resembling the other environments in which LAB and yeasts co-occur. In addition to LAB and yeasts, sourdoughs harbor acetic acid bacteria, whose role in sourdoughs is not well known (Minervini *et al.*, 2014). Cereal flour in sourdoughs is a rich source for amino acids, carbohydrates, fatty acids, minerals and vitamins, and contains also endogenous enzymes that help hydrolyze the nutrients for facilitated utilization (De Vuyst *et al.*, 2014). However, the hydrolysis rate may limit the nutrient abundance. The flour is the major source for LAB and yeasts, but besides flour they can originate from the water, baking equipment and surrounding environment (Minervini *et al.*, 2015). Flour type can affect the microbiota formation especially on account of substrate-derived enzymes, but the most typically used cereals wheat and rye do not exhibit characteristic differences in their microbiota (Gänzle, 2014).

Commercial defined starter cultures that can include both LAB and yeasts are often applied in making of sourdoughs (Brandt, 2014). These starter cultures naturally affect the formation of the sourdough microbiome, even though the starter culture species do not always remain in the community (Smid and Lacroix, 2013). Formation of the sourdough microbial community occurs during the first week of sourdough fermentation, after which the species composition of the community typically stabilizes (Ercolin*i et al.*, 2013).

The formation and stability of sourdough microbiome is also affected by abiotic factors (Minervini *et al.*, 2014). Some key factors include the fermentation temperature and initial pH. Sourdough LAB have an optimum temperature range of 30 to 40°C whereas yeasts prefer temperatures of 25 to 27°C. Thus, lower fermentation temperatures favor yeast growth in sourdough. Typical pH range for traditional sourdoughs is from 3.5 to 4.3, which suits the demands of dominant microbes (i.e. LAB and yeasts) present in sourdoughs. When the initial pH is close to 6, the growth of LAB species is favored, whereas pH below 5 favor yeast growth whilst LAB growth can be completely inhibited. There is a high diversity within genera, species and strains, so the selection affects not only the ratio of LAB and yeasts, but also the ratio of different genera. For example, homofermentative LAB thrive in higher temperatures better than heterofermentative LAB, especially when combined with high dough yields (DY = dough mass x 100 divided by flour mass) of softer sourdoughs. Overall, firm doughs with lower DY favor the growth of yeasts. Fermentation time particularly affects the ratio of differently stress-resistant LAB, and storage temperature adds cold stress as selective pressure. Process factors such as number of back-slopping steps and kneading mechanisms can also affect the microbiota, especially through disturbance that introduces more oxygen to the environment, favoring the growth of more aerobic species.

The composition of microbial communities can be determined using both culture-dependent and culture-independent methods. Widely used identification methods that rely on cultivation of microbes include MALDI-TOF mass spectrometry (Sandrin *et al.*, 2013), selective plating, and a range of biochemical and phenotypic assays, such as catalase tests and Gram staining (Franco-Duarte *et al.*, 2019). However, if microbes that cannot be cultured in laboratory conditions are present in the community, they are not detected by these methods. Even these uncultivable microbes can be identified with culture-independent approaches. Currently this approach is mainly based on next-generation sequencing (NGS). Most established NGS approaches for taxonomic profiling are amplicon sequencing, typically with 16s rRNA as amplicon for bacteria and internal

transcribed spacer (ITS) for fungi, or whole metagenome shotgun sequencing (WMS), both of which can be classified into the category of metagenomics (Franzosa *et al.*, 2015). There are, however, disadvantages to NGS based methods as well, such as primer biases in target gene amplification (Bellemain *et al.*, 2010; Al-Awadhi *et al.*, 2013), unequal DNA extraction yield (Davis *et al.*, 2019), and incomplete databases for poorly characterized organisms (Franzosa *et al.*, 2015). Since both culture-dependent and culture-independent methods have drawbacks, combining different methods to complement one another is often considered preferable (Franco-Duarte *et al.*, 2019).

Analyzing multiple samples from different phases of the fermentation process can produce a great deal of information on community stability and its formation as well. Same approach can be applied to evaluating the microbial load of the sample over time by quantitative methods. Quantification of microbial cells is commonly assessed using flow cytometry, quantitative PCR (qPCR), or the spike-in method which makes use of 16S rRNA gene sequencing (Stämmler *et al.*, 2016; Vandeputte *et al.*, 2017)

Aforementioned approaches have been applied in an attempt to reveal the structure of a typical sourdough microbiome. This has led to the conclusion that there is neither a definite relationship between the sourdough and its associated microbiota (De Vuyst *et al.*, 2014), nor the sourdough microbiota and its geographical location (De Vuyst *et al.*, 2017). An analysis of 583 previously reported sourdoughs shed light on the number and diversity of LAB and yeast species in said ecosystem, highlighting the most common sourdough species (van Kerrebroeck *et al.*, 2017). In 527 cases, LAB species diversity was reported. Based on the analysis, a single sourdough is inhabited by on average 2.0 LAB species, most often more than one. The most common representatives of sourdough LAB were *Lactobacillus sanfranciscensis* (47%) and *Lactobacillus plantarum* (43%). Present in 12 to 17% of the sourdoughs were *Lactobacillus brevis*, *Pediococcus pentosaceus*, *Lactobacillus paralimentarius* and *Lactobacillus fermentum*. Based on these results, LAB in sourdoughs are mostly lactobacilli. Less predominant species belong to weissellas, pediococci and leuconostocs, and finally subdominant lactococci, enterococci and streptococci. In respect of yeast diversity from 394 sourdoughs, it was revealed that *Saccharomyces cerevisiae* was the most common species; it was present in 68% of the sourdoughs. This could be expected due to its common use as a starter culture, causing it to be abundant in the bakery environment as well. Other common yeast species, present in 4 to 20% of the sourdoughs, were (in declining order of

prevalence) *Candida humilis*, *Pichia kudriavzevii*, *Torulaspora delbrueckii*, *Wickerhamomyces anomalus* and *Candida glabrata*. There were on average 1.3 yeast species found from one sourdough, in most cases either one or two species at once.

Interactions between species drive the microbial community assembly and a better understanding of these interactions is required in order to rationally manipulate microbial communities (Abreu and Taga, 2016). Inter-species interactions are traditionally classified by the outcome that a pairwise interaction produces on the organisms involved: positive, negative or neutral (see **Table 1**). The interaction type that has been studied most in microbiology is beneficial mutualism (Zuñig*a et al*., 2017). However, the knowledge of interaction outcome by itself does not elucidate any interaction mechanisms. Interactions are prone to change over time owing to changing parameters, such as nutrient availability (Zuñiga *et al.*, 2017).

Pairwise interaction models have been applied to predicting fates of multispecies microbial communities. Although some limitations remain due to addition of other organisms and abiotic factors possibly changing pairwise interactions (Momeni *et al.*, 2017; Pacheco and Segrè, 2019), these models are considered useful and comparatively reliable in making predictions for outcomes in more complex communities as well (Friedman *et al.*, 2017). For the purpose of creating reliable community models, both discovering interactions and identifying the mechanisms behind them are pivotal.

Table 1. Outcomes of ecological interactions for interaction partners

Ecological interaction	Outcome
Mutualism / Synergism	+/+
Commensalism	+/0
Amensalism	-/0
Predatism / Parasitism	+/-
Competition	-/-
Neutralism	0/0

+ for positive, - for negative and 0 for neutral outcome

There are two main approaches in identifying microbial species interactions: culture-dependent and culture-independent. Culture-dependent experiments traditionally include comparing growth in co-cultures to single cultures or using spent (i.e. conditioned) medium assays. Physical contact takes place in co-cultures, which is not the case for spent medium assays. In spent medium assays one species is grown in the cell-free filtrate of another culture. These culture-dependent assays are

typically performed with isolates from the environment of interest. Samples of the environment can also be directly used to study the species co-occurrence in a culture-independent manner. Although co-occurrence is an indirect indication of interaction, metagenomics cannot reveal the interaction mechanisms but merely poses hypotheses on potentially interacting species. Similarly, hypotheses on interactions can be derived from community metagenome also revealing the functional profile of the microbial consortia. Metatranscriptomics can be used for uncovering the expressed genes, and metaproteomics the protein content (Abreu and Taga, 2016; Zuñig*a et al*., 2017). Singularly they provide quite limited insight into the species interactions, but when data from these multi-omics approaches is combined with computational tools, models can be produced. Model simulations facilitate deriving testable hypotheses on the species interactions within the microbiome. For testing the hypotheses, culture-dependent methods and experimental model systems are essential (Abreu and Taga, 2016). Microbial communities of fermented foods make up ideal experimental model systems in being natural, medium-sized, and containing mainly laboratory cultivable species, yeasts and LAB in particular (Wolfe and Dutton, 2015).

Interaction types of LAB and yeasts in their most studied environment, in fermented foods, are predominantly commensalism or mutualism, typical for stable communities (Sieuwerts *et al.*, 2018). Competition in these communities is not uncommon. However, in stable LAB-yeast communities, niche separation in the utilization of main carbon sources is typical (Gobbetti *et al.*, 1994; Iacumin *et al.*, 2009; De Vuyst *et al.*, 2009). Particularly, the niche separation between maltose-positive and maltose-negative species allows forming stable and non-competitive associations, such as the interactions between the maltose-positive *L. sanfransiscensis* and the maltose-negative yeasts *C. humilis* or *Kazachstania exigua* in sourdoughs (De Vuyst *et al.*, 2009). Stable associations are also formed between species that are able to utilize several carbohydrates, such as between the common sourdough species *L. plantarum*, *L. sanfransiscensis* and *S. cerevisiae* (Iacumin *et al.*, 2009; De Vuyst *et al.*, 2009).

Various chemical bases for LAB and yeast interactions have been discovered in addition to carbohydrates metabolism (Gobbetti *et al.*, 1994): from (de-)acidification of the environment (Stadie *et al.*, 2013; Sieuwerts *et al.*, 2018) and cross-feeding or exchange of metabolites (Narvhus and Gadaga, 2003; Stadie *et al.*, 2013; Ponomarova *et al.*, 2017) to the non-proteinaceous yet unidentified factor secreted by a yeast that promotes LAB growth (Sieuwerts *et al.*, 2018). The chemical interactions between LAB and yeasts also affect the end-products, typically fermented

foods. For example, interactions that lead to the production of antagonistic compounds such as bacteriocins have led to improved shelf-life, and interactions where secondary fermentation pathways are stimulated have affected flavor formation (Alexandr*e et al.*, 2004; Sieuwerts *et al*., 2018). Also, interactions beyond the aforementioned have been reported, such as physical interactions like the flocculation of yeast cells and the formation of biofilms (Bartle *et al.*, 2019). Other types of interactions are less frequent, although some have been discovered, such as a case where LAB and yeast interaction in water kefir could be classified as parasitism (Leroi and Pidoux, 1993). However, positive interaction types mutualism and commensalism are widespread in LAB-yeast communities, and they are the most prominent in regard to applications, such as starter culture optimization (Smid and Lacroix, 2013; Stadie *et al.*, 2013; Penido *et al.*, 2018).

So far, positive interactions between LAB and yeasts have been observed as enabled or increased growth mainly in conditions where availability of carbohydrates or amino acids has been limited (Gobbetti *et al.*, 1994; Ponomarova *et al.*, 2017; Sieuwerts *et al.*, 2018). Such starvation conditions are known to lead to changes in gene expression (Guerzoni *et al.,* 2013) and observations like interactions in said conditions are not be relevant in nutritionally rich environments, such as the common natural environments of LAB and yeasts.

Sourdoughs are nutritionally rich environments where LAB and yeasts co-occur naturally. Revealing LAB-yeast interactions would not only allow improved control of fermentation processes and food safety (Smid and Lacroix, 2013) but also lead to extrapolating the revealed information to more complex communities (Wolfe and Dutton, 2015) and development of novel applications (Tshikantwa *et al.*, 2018; Pacheco and Segrè, 2019). When the interactions are observed in defined growth conditions as opposed to complex undefined conditions, the obtained information is easier to interpret (Zhang *et al.*, 2009) and thus, more readily applicable for creating interaction networks and eventually applications (Tramontano *et al*., 2018; Tshikantwa *et al*., 2018). In this study, the interactions of LAB and yeasts were screened in nutritionally rich chemically defined medium conditions. Species were isolated from rye, wheat and rye-wheat sourdoughs and characterized in both isolation and for LAB-yeast interactions.

Materials and methods

Isolation of LAB and yeasts

LAB and yeast strains were isolated from three types of laboratory-prepared sourdoughs: rye, wheat and rye-wheat mixture (see **Supplementary material** for sourdoughs). Sourdoughs were diluted in sterile water and spread dilution plates 10^{-4} to 10^{-8} were prepared. LAB from rye and rye-wheat mixture sourdoughs were realized in De Man, Rogosa and Sharpe (MRS) agar plates supplemented with 10 µg/ml cycloheximide with incubation at 30°C under anaerobic conditions for 2 to 4 days. LAB from wheat sourdough were realized in MRS agar plates with no antibiotics at 30°C grown under aerobiosis for 3 days. After incubation, 10 isolates were picked on fresh MRS plates based on colony morphology. To obtain more reliable pure cultures of each isolate, a single colony was diluted in 1 ml of sterile water and 10^{-3} and 10^{-4} dilutions were plated, then this procedure was repeated for a single colony picked from either dilution plate. Standard Gram staining and catalase tests were performed for phenotypical testing of suspected LAB isolates.
Yeasts were realized in yeast extract peptone dextrose (YPD) agar supplemented with 10 µg/ml chloramphenicol. Plates were incubated at 25°C for 2 to 5 days. After incubation, 12 isolates were picked on fresh YPD plates based on colony morphology. To obtain purified cultures of yeast isolates, single cells were separated using a dissection microscope following manufacturer's instructions (MSM 400, Singer Instruments).

Genotypic characterization of LAB and yeast isolates

Primary characterization of the isolates was performed using MALDI-TOF MS (matrix-assisted laser desorption/ionization mass spectrometry) biotyper (Bruker) according to manufacturer's protocol. LAB isolates that could be identified to a species level with high-confidence identification score (≥ 2,0) were not included in further identification steps. Remaining isolates were identified by amplicon sequencing. The genomic DNA was extracted from colonies picked from plates using the FastDNA® Spin Kit for Soil according to the manufacturer's protocol. The extracted DNA was amplified using the 16S primers (BSF 8/20 AGAGTTTGATCCTGGCTCAG and BSR 1541/20 AAGGAGGTGATCCAGCCGCA) for bacteria and with ITS primers (ITS1F TCCGTAGGTGAACCTGCGG and ITS4R TCCTCCGCTTATTGATATGC) for yeasts. PCR products were treated with MyTaq™ Red Mix (Bioline). PCR products of the isolates were sent for DNA sequencing at Microsynth Seqlab, Germany. Isolates were identified to a species level

using the obtained sequences with the Blast tool (Basic Local Alignment Search Tools Nucleotide) from NCBI (National Center for Biotechnology Information).

Chemically defined culture media

A chemically defined rich culture medium (ZMB) was designed with sugar composition mimicking the sugar monomer availability in sourdough. The recipe of a chemically defined rich culture medium supporting high cell density growth of several bacteria (Zhang-Mills-Block1 from Zhang *et al.*, 2009) was modified based on an HPLC analysis of sourdough (Sieuwerts *et al.*, 2018), replacing glucose as a sole carbohydrate source with galactose, fructose, maltose and galactose in appropriate ratios maintaining the total carbohydrate concentration. In addition, four medium variants were prepared from the basic ZMB recipe: two vitamin variants with either riboflavin or folic acid left out and two sugar variants with either glucose or maltose left out. Total volume and sugar amount were kept the same between the medium variants. All media had their pH set to 7.0 with 5 M hydrogen chloride (HCl) before adjusting to final volume with sterile water. From the basic ZMB medium, also aliquots where pH was set to 3.0 and 5.0 with HCl were prepared. Finally, the media were sterilized through a 0.2 µm filter. Complete culture media were stored at 4°C.

Culture conditions and preparation of overnight cultures

After isolation and identification, all LAB and yeast isolates were cultured at 30°C under aerobiosis without shaking. MRS agar plates for LAB and YPD for yeasts were used. Cultures for experiments were started by inoculating 4 ml selected fresh culture medium with plate culture in 24-well culture plates, which were then covered with sterile breathable rayon film. Second inoculation to align the growth of the cultures was done on the following day with start OD_{600} of 0.2 based on spectrophotometer measurements. These cultures were grown overnight and then considered ready to use for various growth assays. Culture media in use was mostly defined medium ZMB or its variations. LAB isolates that did not grow on defined medium were grown on MRS broth to produce overnight cultures.

Turbidimetric assays

Turbidity was determined by optical density (OD) measurements at 600 nm. Single measurements from cultures were taken using spectrophotometer Ultrospec 2100 Pro (GE Healthcare). Samples were diluted with sterile water to obtain an OD_{600} readout between 0.1 to 0.4. These measurements

were used to check for growth in cultures and calculate required inoculation volume for specific starting ODs.

Bioscreen C automated microbiological growth analyzer (Oy Growth Curves Ab Ltd.) with BioScreener™ measurement software (v3.0.0) was used for periodical analysis of turbidity. Bioscreen Honeycomb 2 plates, 100-well microtiter plates were used with 200 µl sample volume per well. Using overnight cultures, the starting OD_{600} for each well was set at 0.01 or 0.05 depending on experiment. Four technical replicates of each sample were used. The runs were set for 5 days, with steady temperature at 30°C. Measurements were set to be taken every 30 minutes with shaking at medium speed for 10 seconds before each measurement, shaking stopped 5 seconds prior. After the run was complete, raw data was exported from BioScreener™ and used for further analyses.

Determination of maximum OD and specific growth rate

Maximum OD and specific growth rate for each isolate in different culture medium variants were determined. The raw data from Bioscreen was analysed using Matlab v.9.3.0 (release 2017b). With an in-house script, the natural logarithm of the raw growth data was smoothed and interpolated with the function csaps (cubic smoothing spline) in order to reduce noise in the data. The smoothing parameter was adjusted appropriately to receive a smooth curve; however, the results are not very sensitive to its exact value. Taking the time derivative of the smooth curve and finding its maximum value delivered directly the maximum specific growth rate while the maximum OD was derived similarly from exponential function of the smoothed curve.

Determination of correlation between cell dry weight and optical density

To estimate cell dry weight in relation to OD_{600} measurements specifically for each isolate, 10 ml tubes of either ZMB or MRS were inoculated with plate cultures. The threshold for culture turbidity was set at OD_{600} of 5.0 (Spectrophotometer) for this. Once reached, 6 ml of each culture was divided equally in triplicate pre-dried microcentrifuge tubes that had been weighed beforehand. Aliquots were pelleted and then washed with sterile H_2O twice with centrifugation at maximum speed, after which tubes with open corks containing the pellets were left to dry over two nights at +105°C. Finally, the tubes containing cells were weighed and the weight of dried tubes was subtracted from each. The cell dry weight was estimated in grams per liter as averages of triplicates. The OD_{600} measurement at the time of gathering was used to estimate specific relations for each isolate between the turbidity and cell dry weight.

Determination of specific uptake and production rates

To determine the uptake rates of sugars and production rates of ethanol and lactic acid, supernatant samples were analyzed by Alliance High Throughput HPLC Systems (Waters Corp) along with QuickStart Empower 3 software. Supernatants were gathered from three time points during exponential growth phase for each isolate that grew in ZMB medium. Collection time points were determined by inspecting growth curves created on Bioscreen, and OD_{600} measurements were also taken prior to each collection to ensure that they were within the range of exponential growth. Three biological and two technical replicates for each strain were included. Eluent used in HPLC run was 5 mM sulfuric acid (H_2SO_4). Mixtures of compounds of interest (ethanol, lactic acid, maltose, glucose, fructose and galactose) at concentrations of 0.25, 0.5, 0.75, 1.0, 2.5, 5.0, 8.5 and 10.0 g/l with 5mM H_2SO_4 were prepared to use as standards alongside pure ZMB medium. Technical replicates were averaged.

The specific uptake and production rates were calculated using the following equation:

$$q = \frac{\Delta c}{\Delta t \cdot x}$$

where Δc is the difference in concentration (g/l) of compound c in the culture supernatant and Δt the difference in time between subsequent sampling time points. x is the biomass concentration (g cdw/l) at the arithmetic mean time point between the sampling times estimated from isolate specific OD_{600} - cell dry weight correlation and linear regression between $\ln(OD_{600})$ at sampling points and sampling time. R-squared value of the linear regressions were evaluated for confirming that the sampling points fell to the the exponential phase of growth (R-squared ≥ 0.85) and deviating intermediate OD_{600} measurements or biological replicates were discarded.

Spent medium assays

Spent media were produced by collecting supernatants from all isolates that grew on ZMB at two stages of their growth: at the exponential phase and at the stationary phase. Overnight cultures were used to inoculate 11 ml of ZMB medium at start OD_{600} of 0.05, two parallel tubes were used. Collection time points were determined by growth curves from Bioscreen runs with the same start OD, also OD_{600} of cultures was checked prior to collection. Supernatants of parallel cultures were pooled together at the harvest and filtered through 0.2 µm filters to obtain sterile spent media. The spent media were stored at +4°C. The spent medium growth assays were performed with Bioscreen by inoculating spent media with overnight culture at start OD_{600} of 0.01.

NMR spectroscopy

Samples for NMR were prepared by mixing 540 µl of sample and 60 µl of D_2O (Euriso-Top, St-Audin Cedex, France) in 5 mm NMR tubes. The NMR spectra were recorded at 25°C on a 600 MHz Bruker Avance III NMR spectrometer (Bruker Biospin) equipped with QCI H-C/N/P-D cryogenically cooled probe head and SampleJet automated sample changer. The water signal was suppressed by volume selective presaturation (so-called noesy-presat) using Bruker's pulse program noesygppr1d. Number of scans was 128. The spectra were processed by Bruker's software Topspin 3.6.2 and assigned using the spectral libraries of ChemAdder (Spin Discoveries), Madison Metabolics Consortium Database (http://mmcd.nmrfam.wisc.edu/) and human metabolome database HMDB (https://hmdb.ca/).

Co-culture stability testing

Stability of pairwise co-cultures was tested by inoculating 4 ml of fresh ZMB culture medium with overnight cultures to start OD_{600} of 0.025 each. Every 48 hours 20 µl was transferred to 2 ml of fresh medium. Two biological replicates for each pairwise culture were used. Pairs consisted of one LAB isolate and one yeast isolate. Cultures were inspected by light microscopy to observe which cell types were present.

Results

LAB and yeast isolate set from rye, wheat, and rye-wheat sourdoughs

Species were isolated from rye, wheat and rye-wheat sourdoughs and identified to the species level (see **Table 2**). Five LAB isolates identified by MALDI-TOF were representatives of the species *L. plantarum*. LAB isolates identified by 16s rRNA sequencing were *Lactobacillus rossiae* (3 isolates) and *Pediococcus parvulus* (2 isolates) strains. Based on ITS sequencing, yeast isolates were *W. anomalus* (6 isolates) and *T. delbrueckii* (6 isolates) strains. Multiple yeast strains were included in the sourdough isolate set in case their growth profiles were distinct (**Table 2**). Two distinctive *W. anomalus* were identified whilst the *T. delbrueckii* isolates were indistinguishable. All LAB isolates were included, except for a *P. parvulus* isolate with a relatively low identification score. Finally, the sourdough isolate set was further augmented with yeasts *P. kudriavzevii, P. fermentans* and *K. marxianus* previously isolated from sourdoughs established on same flour types (see **Supplementary material**).

Table 2. Identified sourdough LAB and yeast isolates

Isolate No.	Identified species	Isolate code[†]	BLAST ID %	MALDI-TOF ID score[††]	Sourdough flour type
1	*Lactobacillus plantarum*	L.p2	NI[†††]	2.26	Rye
2	*Lactobacillus plantarum*	L.p3	NI	2.26	Rye
3	*Lactobacillus plantarum*	L.p4	NI	2.30	Rye
4	*Lactobacillus rossiae*	L.ros5	99.8	1.82	Rye
5	*Wickerhamomyces anomalus*	W.a2	99.8	2.05	Rye
6	*Wickerhamomyces anomalus*	-	99.8	1.84	Rye
7	*Wickerhamomyces anomalus*	-	100.0	2.03	Rye
8	*Torulaspora delbrueckii*	-	100.0	NI	Rye
9	*Torulaspora delbrueckii*	-	100.0	NI	Rye
10	*Pediococcus parvulus*	-	99.1	NI	Wheat
11	*Pediococcus parvulus*	P.par	99.6	NI	Wheat
12	*Wickerhamomyces anomalus*	-	100.0	1.70	Wheat
13	*Wickerhamomyces anomalus*	W.a1	100.0	1.78	Wheat
14	*Wickerhamomyces anomalus*	-	100.0	1.72	Wheat
15	*Lactobacillus plantarum*	L.p7	NI	2.30	Rye-Wheat
16	*Lactobacillus plantarum*	L.p8	NI	2.35	Rye-Wheat
17	*Lactobacillus rossiae*	L.ros9	99.5	1.87	Rye-Wheat
18	*Lactobacillus rossiae*	L.ros10	99.6	1.89	Rye-Wheat
19	*Torulaspora delbrueckii*	-	100.0	1.70	Rye-Wheat
20	*Torulaspora delbrueckii*	-	100.0	NI	Rye-Wheat
21	*Torulaspora delbrueckii*	T.del	100.0	NI	Rye-Wheat
22	*Torulaspora delbrueckii*	-	100.0	NI	Rye-Wheat

[†] Isolate code introduced for the distinguishable yeast strains and all LAB except for isolate no. 10.
[††] Highest score from parallel samples was included. Score values ranging from 0.00 to 1.69 indicate no identification, 1.70 to 1.99 low-confidence identification and 2.00 to 3.00 high-confidence identification (from Bruker MALDI Biotyper Identification Results).
[†††] NI = Not identified with method in question.

Growth performance of isolates on defined culture medium

The growth of *L. plantarum* (Ponomarov*a et al*., 2017), *L. rossiae* (Boguta *et al.*, 2014), *K. marxianus* (Fonseca *et al.*, 2013), *P. kudriavzevii* (Toivari *et al.*, 2013), *T. delbrueckii* (Visser *et al.*, 1990) and, *W. anomalus* (Fredlund *et al.*, 2002) belonging to the set of species isolated in the rye, wheat, and rye-wheat sourdoughs, have previously been characterized on defined medium. However, no previous reports of the growth of *P. parvulus* or *P. fermentans* in defined growth media existed to the best of our knowledge. For this study, a defined medium was designed based on the ZMB growth medium developed by Zhang *et al.*, (2009) in order to support growth of several bacteria, including demanding LAB. The recipe for the medium was modified to have its carbohydrates content resemble that of sourdough's (Sieuwerts *et al.*, 2018). All the sourdough isolates in the defined set (**Table 2**) were cultured in the prepared defined medium.

All yeast isolates reached substantial growth in the defined medium ZMB. Yeast isolates P.fer, K.mar and T.del (**Table 2**) had single exponential growth phase, whereas W.a1, W.a2 and P.kud (**Table 2**) had multiple growth phases (**Figure 1**) indicating sequential utilization of nutrients (Chu, 2015). Out of LAB isolates, growth of all *L. plantarum* isolates was supported by ZMB, whereas no growth beyond the threshold considered here as growth (maximum $OD_{600} \geq 0.25$) could be detected for any *P. parvulus* or *L. rossiae* isolates.

Growth of the *L. plantarum* and yeast isolates was further analyzed in ZMB medium variants, where either glucose, maltose, riboflavin or folic acid had been left out. This was in order to screen for auxotrophies regarding vitamins or the capability to utilize only a selected carbohydrate source. There were no substantial changes observed in maximum ODs or specific growth rates between the variants for any isolate (**Figure S1**). Overall, yeasts grew faster and reached higher maximum ODs than LAB.

To assess factors for the differences in the maximum specific growth rates of isolates, specific sugar uptake rates during exponential growth phases were determined. Uptake rates and ethanol production for yeast isolates are shown in **Table 3**. While high variance in biological replicates did not allow for reliable ranking of LAB isolates, it could be concluded that yeast maximum specific growth rates were not explained by the maximum specific glucose uptake rates (**Figure S1**). The yeast with highest sugar uptake rates was *P. fermentans* isolate, whilst based on growth it was only the 5^{th} fastest. On the other hand, the fastest growing isolate, K.mar, had the lowest glucose uptake rate and second lowest uptake rate for fructose/galactose and production rate of ethanol. None of the yeast isolates consumed maltose while glucose was abundantly present. Co-consumption of other sugars at low rate was observed. Fructose and galactose consumptions could not be distinguished, but due to relatively low amount of galactose in the medium, at least fructose is likely to be co-consumed. Specific production rates of ethanol and lactate were also determined in the first exponential growth phase **Table 3**. *P. fermentans* isolate had the highest specific ethanol production rate of the yeasts. Lactic acid production was observed in case of all yeast isolates, most notably *T. delbrueckii* isolate. Lactic acid production rate was observed to be higher for LAB isolates than for most yeast isolates, or on the same level with *T. delbrueckii* (data not shown).

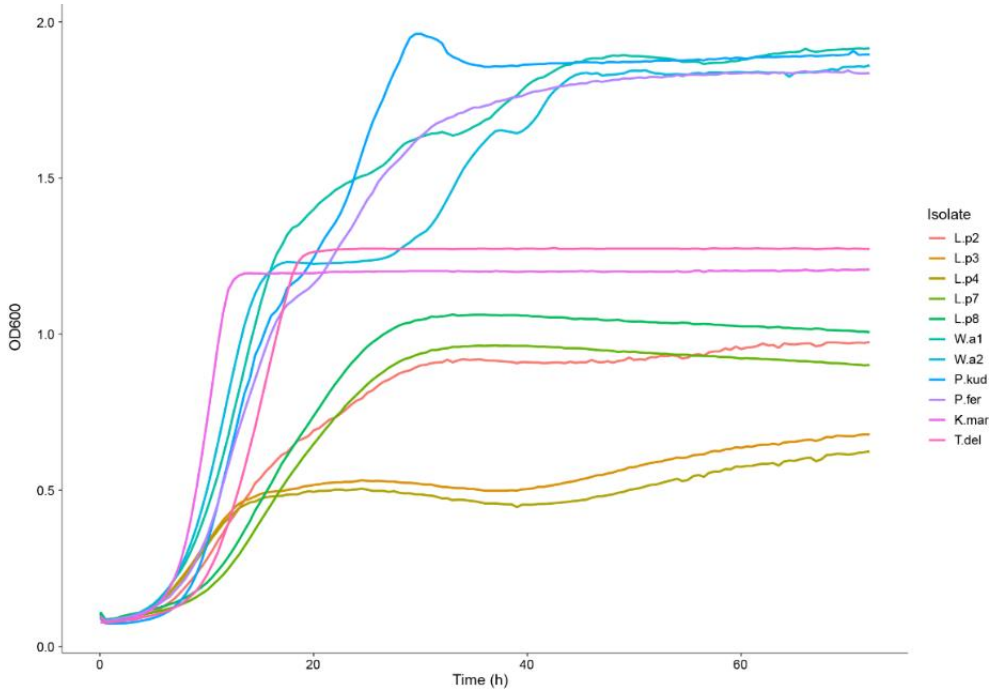

Figure 1. Growth profiles of LAB and yeast isolates in the chemically defined medium OD_{600} over time for each isolate whose growth was supported by the defined medium (ZMB).

Table 3. Selected uptake and production rates of yeast isolates in exponential growth phase in the chemically defined medium

Isolate	Specific glucose uptake rate (g/g CDW h)	Specific fructose/galactose uptake rate (g/g CDW h)	Specific lactic acid production rate (g/g CDW h)	Specific ethanol production rate (g/g CDW h)
W.a1	-2.31	-0.22	0.17	0.52
W.a2	-2.65	-0.31	0.26	0.88
P.kud	-3.47	-0.35	0.43	1.18
P.fer	-5.20	-0.66	0.60	1.68
K.mar	-2.13	-0.29	0.24	0.74
T.del	-3.62	-0.63	2.07	1.06

Yeast metabolism has positive and negative effects on LAB

The impact of yeast metabolism on the LAB isolates was investigated with spent-medium (SM) assays. This was done to screen for possible interactions, such as growth promotion or inhibition. Exponential growth phase SM was used for assessing the effect of yeast byproducts on LAB growth, and stationary phase SM for determining the ability of LAB isolates to grow solely on the leftovers of yeasts. Exponential SM was collected during the first exponential growth phase of

each yeast, and the stationary SM was collected after 24 hours (K.mar and T.del) or 48 hours (W.a1, W.a2, P.kud and P.fer). Stationary SM of T.del was dismissed as an outlier due to low OD at collection.

The maximum OD_{600} the LAB isolates reached in yeast SM was compared to fresh defined medium ZMB (**Figure S2**). Neither the exponential nor stationary phase yeast SM supported growth of *P. parvulus* and *L. rossiae* isolates that would not grow on fresh ZMB, except for a single *L. rossiae* isolate. L.ros5 isolate was found to grow on exponential phase yeast SM, yet the growth was poorly reproducible (**Figure 2a**). However, L.ros5 growth was only observed in SM and not once in the fresh ZMB. Further, the absence of yeast contamination was confirmed with light microscopy and the lower specific growth rate of L.ros5 in SM rules out a cross-contamination by a LAB (**Figure 2b**). The potential growth-enabling effect of yeast acidified environment on L.ros5 was examined by culturing L.ros5 in ZMB at pH 3.0 and 5.0. However, no growth was observed in low pH media (data not shown). The finding suggests that yeasts produce a yet-unknown compound in the exponential growth phase that supports growth of the *L. rossiae* isolate (L.ros5).

As for *L. plantarum* isolates, growth was generally supported better in exponential phase yeast SM than in stationary phase SM (**Figure S2**), which could be expected due to more nutrients remaining in the exponential SM. There was variation between maximum ODs that the LAB isolates reached between SM from different yeasts. Some of the variance was considered to be due to SM gathering having occurred at varying ODs of the yeast. However, there were no cases where the growth of *L. plantarum* isolate in exponential SM was notably improved when compared to growth in the fresh ZMB. *L. plantarum* isolates reached also substantial growth in all stationary phase SM; in minimum the LAB were able to grow in the stationary phase SM to 44% of OD reached on the fresh medium. As an exception, in the W.a2 stationary phase SM none of the *L. plantarum* isolates grew. However, no growth inhibition by W.a2 on LAB was observed in co-cultures (next section). Thus, it was suspected that the *W. anomalus* isolate (W.a2) had depleted an essential nutrient that *L. plantarum* isolates would require to grow. Therefore, the stationary SM of W.a2 along with the fresh defined medium ZMB were further analyzed by non-targeted NMR spectroscopy -based metabolomics (**Figure 2c**). Stationary SM of K.mar, on which *L. plantarum* isolates reached substantial growth, was used as a control. Main carbohydrate sources glucose and fructose had almost completely been depleted from the stationary SM of W.a2, suggesting that the reason for *L. plantarum* isolates not being able to grow in the particular yeast SM was the absence of preferred

carbohydrates. Low amount of maltose remained in the SM of W.a2. W.a2 had also produced a substantial amount of lactate in the stationary phase SM; increasing initial concentrations of lactate in the environment has been reported to decrease or completely inhibit the growth of *L. plantarum* (Giraud *et al.*, 1991). Higher concentrations of carbohydrates remained in the SM of K.mar, which had supported *L. plantarum* growth. Also, no substantial amount of lactate could be observed in the stationary phase SM of K.mar. Notably, W.a2 had produced some unidentified compounds that were absent in the stationary phase SM of K.mar that may have affected LAB growth (**Figure 2c**).

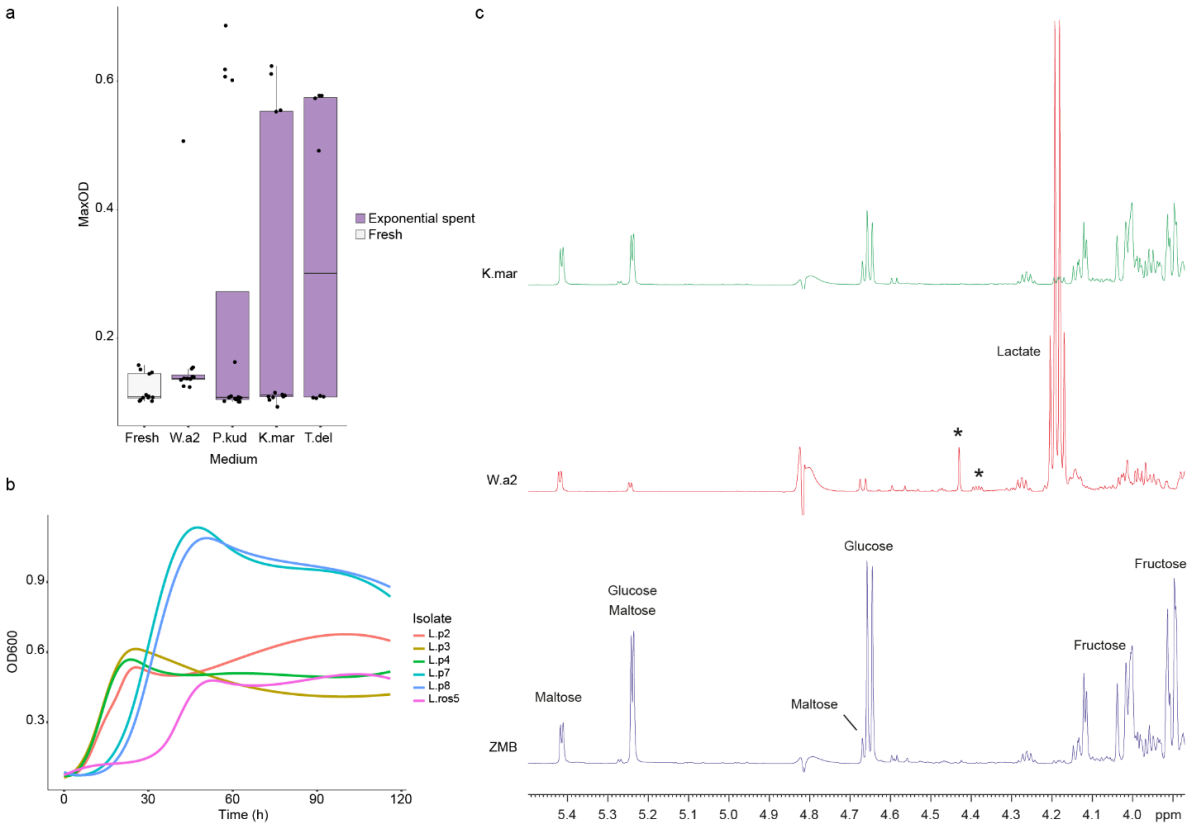

Figure 2. Potential interactions discovered in spent media (SM) a, *L. rossiae* isolate L.ros5 reached higher maximum OD_{600} in SM collected from exponential phase of selected yeast cultures than in fresh defined medium ZMB, although with poor reproducibility. One dot per replicate. **b**, Growth profiles of LAB isolates on exponential phase SM of yeast W.a2. One replicate per isolate included in the graph for clarity. **c**, NMR Spectroscopy image of the chemically defined medium ZMB and the stationary phase SM of isolates K.mar and W.a2. * for unknown compounds not present in ZMB.

Selected LAB and yeast isolates formed stable pairwise co-cultures

From the species belonging to the isolate set, the following co-cultures have been reported: *L. plantarum* with the yeast *W. anomalus* (Coda *et al*., 2011) and the yeasts *T. delbrueckii*, *K. marxianus* and *P.kudriavzevii* (Chaves-López *et al.*, 2014), but not with *P. fermentans*. *L. rossiae* and *P. parvulus* have been reported in co-cultures with yeasts but not with any of the species represented by sourdough isolates in this study (Martens *et al.*, 1997; Winters *et al.*, 2019).

Isolate pairs for co-cultures were formed so that both isolates originated from the same type of sourdough (e.g. rye sourdough). Only the LAB isolates of the same species with distinctive growth profiles (**Figure 1**) were included into the assay. The stability of the pairwise co-cultures was monitored for two weeks with passaging to fresh ZMB medium every other day (six transfers in total). The co-cultured isolate pairs and the outcomes of the stability experiments are shown in **Table 4**. Stable co-cultures were considered established if both LAB and yeast cells could be observed with light microscopy after two weeks (**Figure S3**). In all the pairwise co-cultures where stable co-culture was not established, yeasts had outgrown LAB. There were no cases where only LAB remained present or where neither of the isolates would be present after two weeks of continuous transfers to fresh medium. Interestingly, while the LAB isolates not able to grow on ZMB in pure cultures did generally also not form stable co-cultures with yeasts, the *L. rossiae* isolate L.ros5 formed a stable co-culture with *W. anomalus* isolate W.a2 (**Figure 3a**). Again, the L.ros5 growth reproduced poorly. The *L. plantarum* isolates formed stable co-cultures with yeast isolates every time with no signs of competitive exclusion. *L. plantarum* isolates grew successfully even with isolate W.a2 (**Figure 3b**) which had been observed to consume the primary carbohydrate sources from *L. plantarum* isolates in spent medium assay.

Table 4. Stability of pairwise co-cultures of LAB and yeasts

Pair of sourdough isolates of same origin		
LAB isolate	Yeast isolate	Stability established in co-culture[†]
L.p2	W.a2	+
L.p2	P.kud	+
L.p2	P.fer	+
L.p2	K.mar	+
L.p3	W.a2	+
L.p3	P.kud	+
L.p3	P.fer	+
L.p3	K.mar	+
L.ros5	W.a2	±
L.ros5	P.kud	-
L.ros5	P.fer	-
L.ros5	K.mar	-
P.par	W.a1	-
L.p8	T.del	+
L.ros9	T.del	-

[†] (+) for pairs that formed stable co-cultures, (-) for pairs that did not, (±) for pairs where results reproduced poorly

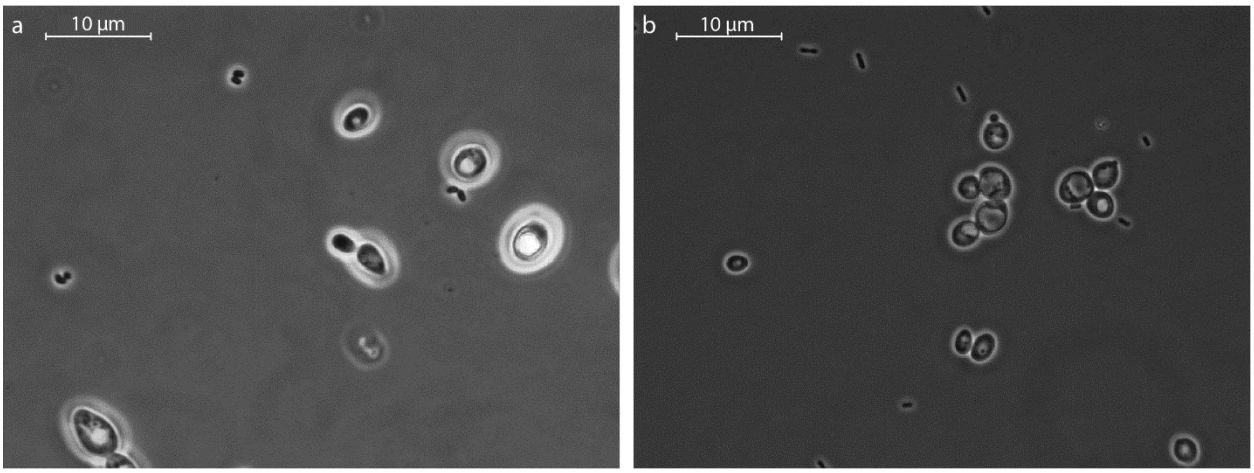

Figure 3. LAB and yeast isolates formed stable communities Light microscopy images of co-culture samples revealed presence of bacterial and yeast cells after six transfers to fresh growth media **a**, Isolates L.ros5 and W.a2 **b**, Isolates L.p2 and W.a2

Discussion

Both LAB and yeast species discovered in the wheat, rye, and wheat-rye sourdoughs were typical sourdough species (Van Kerrebroeck *et al.*, 2017) except for *P. parvulus*. *P. parvulus* appears particularly in beverage fermentations as a spoilage bacterium (Fernandez *et al*., 1996; Miranda-

Castilleja *et al.*, 2016). In case of cereal-based products, using *P. parvulus* as a potential probiotic and protective culture has been looked into (Immerstrand *et al.*, 2010). While other related species belonging to the genus *Pediococcus* are common in sourdoughs (Ercolini *et al.*, 2013; Van Kerrebroeck *et al.*, 2017), this is to our best knowledge the first isolation of *P. parvulus* from said environment.

Even though the number of species discovered per sourdough was somewhat in line with current estimates (Van Kerrebroeck *et al.*, 2017), it is likely that especially the bacterial diversity in the sourdoughs is substantially wider than the isolated species diversity. This study focused on isolating and identifying laboratory cultivable species for interaction screening. With the culture dependent methods used, organisms that require enrichment or specific physiological conditions go undetected (Iacumin *et al.*, 2009). It has been estimated that 99 % of bacteria are unculturable in single cultures, for which one suggested explanation is that many bacteria are dependent on the metabolic activity of other species in their community environment, and not viable when taken out of it (Pande and Kost, 2017).

Discovering and dissecting metabolic interactions is essentially dependent on defined culture medium. In this study a defined complete growth medium was designed for sourdough LAB and yeasts to enable screening for inter-species interactions in rich sourdough-mimicking yet chemically defined conditions. Most of the isolates grew well in the designed defined medium, yeasts faster than LAB. This was, to the best of our knowledge, the first report of characterization of *P. parvulus* and *P. fermentans* in a defined growth medium. Neither *P. parvulus* nor *L. rossiae* isolates grew on the defined complete medium. Pediococci are known as fastidious species (Wade *et al.*, 2019) and the growth of *L. rossiae* was not supported by two distinct defined media in a study by Boguta *et al.* (2014) either. *L. rossiae* has reportedly grown on a semi-defined medium, where a defined medium similar to the one in this study was supplemented with fatty acid and peptide sources (De Angelis *et al.*, 2014), which suggests that these could be the limiting factors. Even though the defined medium designed in this study was rich in amino acids whose absence typically limit LAB growth, many LAB strains tend to also require peptides or vitamin derivatives (Wade *et al.*, 2019) provided by undefined complex media that contain generally peptone, beef extract or yeast extract (Ibrahim, 2013).

Sourdough yeast isolates grew faster on the defined medium than the LAB. However, the *L. plantarum* isolates were able to grow substantially also on the sole leftovers of most yeasts (i.e.

spent medium collected in stationary phase). This suggests that the yeast metabolic byproducts provide a major nutrient reservoir for the LAB. An exception was one *W. anomalus* isolate whose leftovers did not support the growth of *L. plantarum* isolates. It was discovered that the yeast had consumed the majority of the sugars from the culture medium, suggesting that the species require the same carbon sources. *W. anomalus* isolate had also produced notable amounts of ethanol and lactate and some unidentified compounds that might have inhibited LAB growth in the spent medium by presenting too stressful environment. Surprisingly, in co-cultures all *L. plantarum* isolates formed stable communities with *W. anomalus*, and no competitive exclusion or growth inhibition could be noticed. This suggests underlying interaction mechanisms beyond pure competition like partial growth inhibition or niche separation. Stable communities were also formed between *L. plantarum* isolates and all other yeast isolates. Passaging to fresh environment did not allow the yeasts to take over at any time, despite their ability to grow notably faster and reach higher turbidities in the defined conditions. In sourdoughs a slower release of carbohydrate monomers could further stabilize the differences in maximum specific growth rates.

Niche engineering by other species may provide growth enabling nutrients also when all simple essential nutrients are available in rich growth environment. Even though no growth in fresh defined medium could be observed even once, one of the *L. rossiae* isolates reached substantial growth in yeast conditioned medium. However, the growth reproduced poorly: either the *L. rossiae* isolate grew to a similar extent between spent media or not at all. The *L. rossiae* isolate formed also a stable co-culture with the yeast *W. anomalus*, but again with poor reproducibility. A potential cause for poor reproducibility of LAB growth is their general response to multiple stresses in suboptimal conditions leading to unculturability (Papadimitriou *et al*., 2016) and sensitivity to bacteriophages (Garneau and Moineau, 2011). The optimization of culturability is essential for the elucidation of components and mechanisms underlying yeast supporting the growth of *L. rossiae*.

Laboratory culturability of the majority of member species and medium size makes microbial communities of fermented foods excellent natural model systems (Wolfe and Dutton, 2015). Natural model systems are essential for elucidating microbial community assembly and function through inter-species and species-environment interactions (Pacheco and Segrè, 2019). Resolving the mechanisms of interaction is yet limited by availability of chemically defined media for culturing the species (Tramontano *et al.*, 2018). In this study, a chemically defined sourdough mimicking medium was designed, the growth of sourdough isolates was characterized on the medium and potential inter-species interactions were discovered. The species set characterized

provides a resource for revealing fundamental principles of microbial community assembly and function by assembling synthetic microbial communities out of naturally co-occurring species. Elucidating the assembly of microbial communities of LAB and yeast is beneficial not only for food applications but for all other fields where microbial communities play a part.

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Supplementary material

Sourdoughs

Flours used in sourdough preparation were commercial products: Kinnusen Mylly wheat flour and Myllyn Paras rye flour. Three types of sourdoughs were used in the study: wheat, rye and a 1:1 mixture of wheat and rye. The sourdoughs were started with 20 g flour and 20 ml sterile H_2O and incubated at 25°C. Enrichment for each sourdough was performed by feeding with 10 g of appropriate flour and 10 g sterile water daily for two weeks. After this, the feeding continued on a weekly basis for 4.5 months. At this stage, 10 g of each sourdough was transferred to a new sterile beaker with 10 g of appropriate flour and 10 g sterile water. Weekly feeding was continued for 9 weeks until sourdoughs were stored at 0°C. All following isolations were taken within a month.

Previously isolated yeasts

Strains had been isolated at VTT earlier the same year originating from same type of rye sourdough as the ones isolated in this study were. Previous isolates had been identified by ITS sequencing as representatives of species *Pichia kudriavzevii* (isolate code to be used: P.kud), *Pichia fermentans* (P.fer) and *Kluyveromyces marxianus* (K.mar). Isolates had been stored as plate cultures on YPD plates at 4°C.

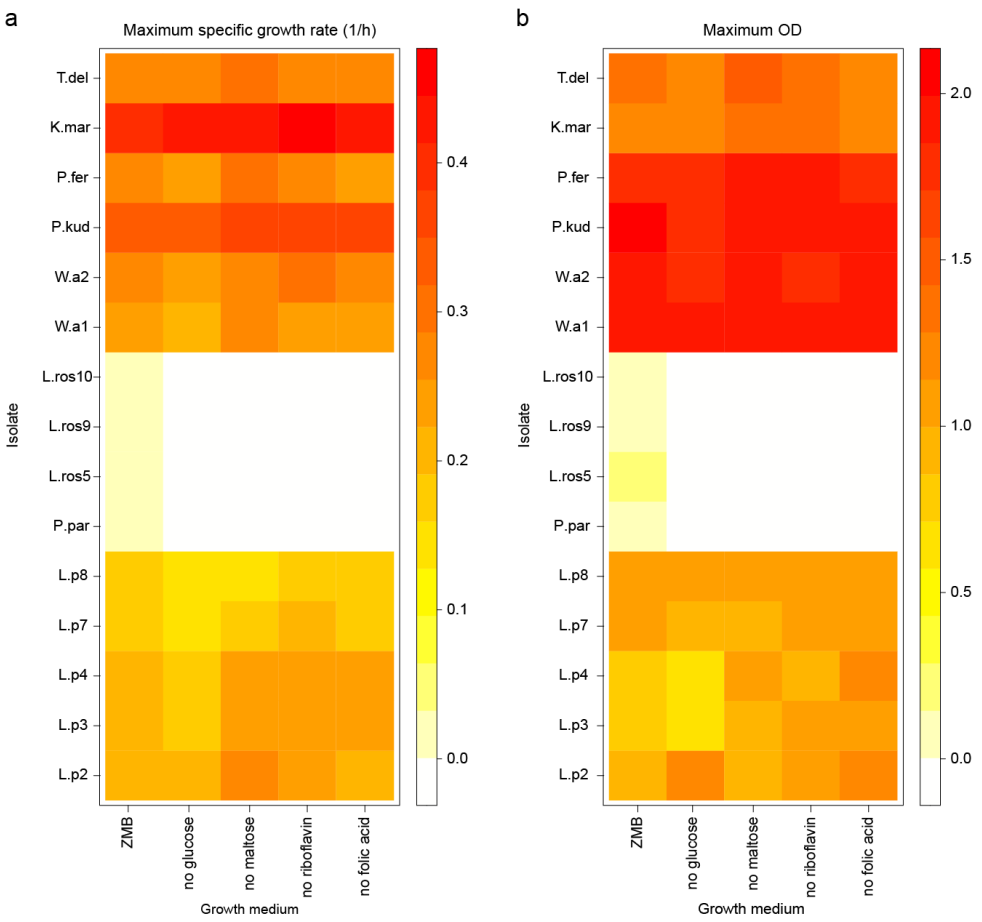

Figure S1. Growth of sourdough isolates in single cultures on defined medium ZMB and its variants a, Maximum specific growth rate (1/h) **b,** maximum OD_{600} (Bioscreen OD) of isolates. White cells for isolates *L. rossiae* and *P. parvulus* in medium variants indicate missing data.

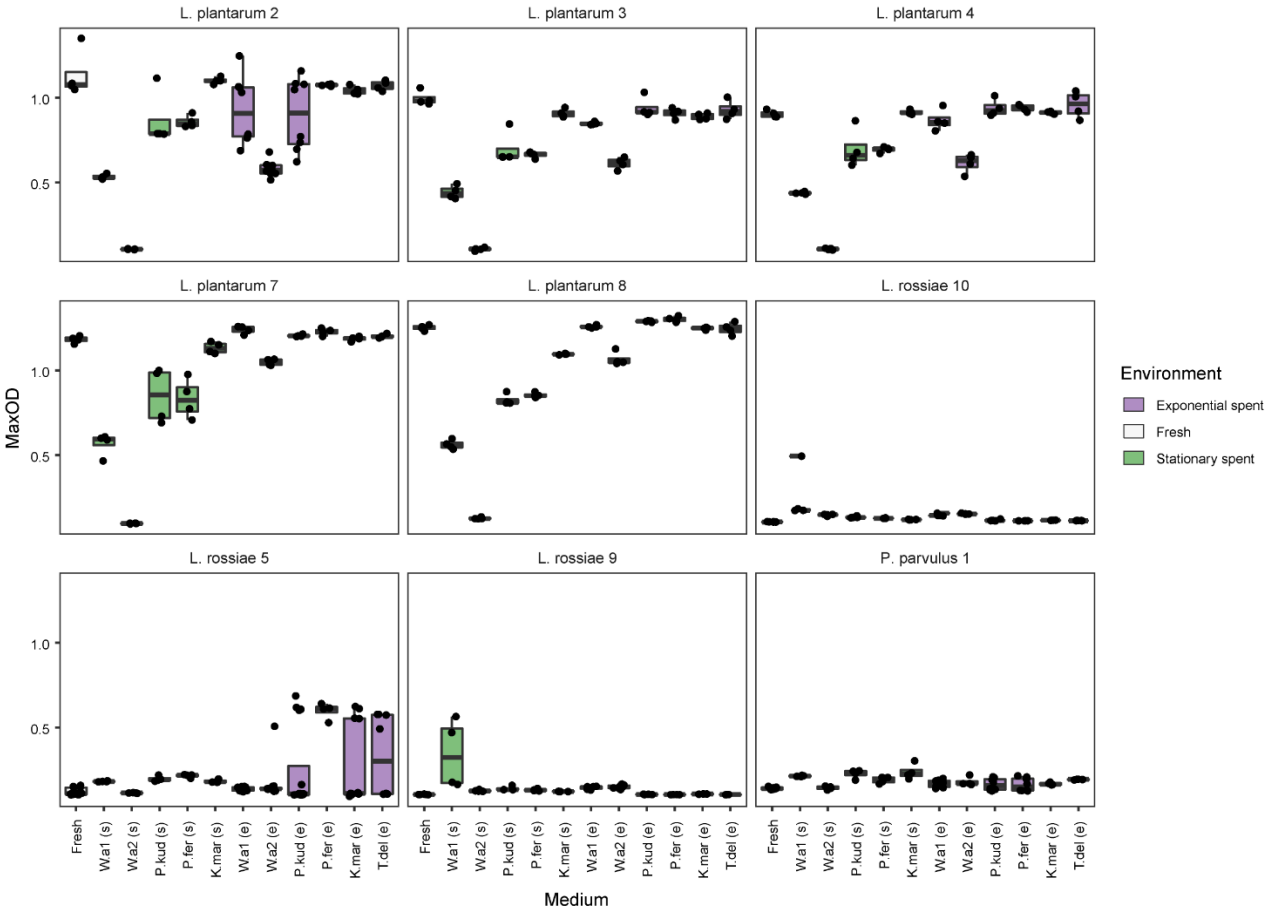

Figure S2. Growth of LAB isolates in spent medium of yeast isolates Comparison of maximum OD_{600} each LAB isolate reached in fresh defined medium (ZMB) and yeast spent media collected at stationary (s) and exponential (e) growth phase.

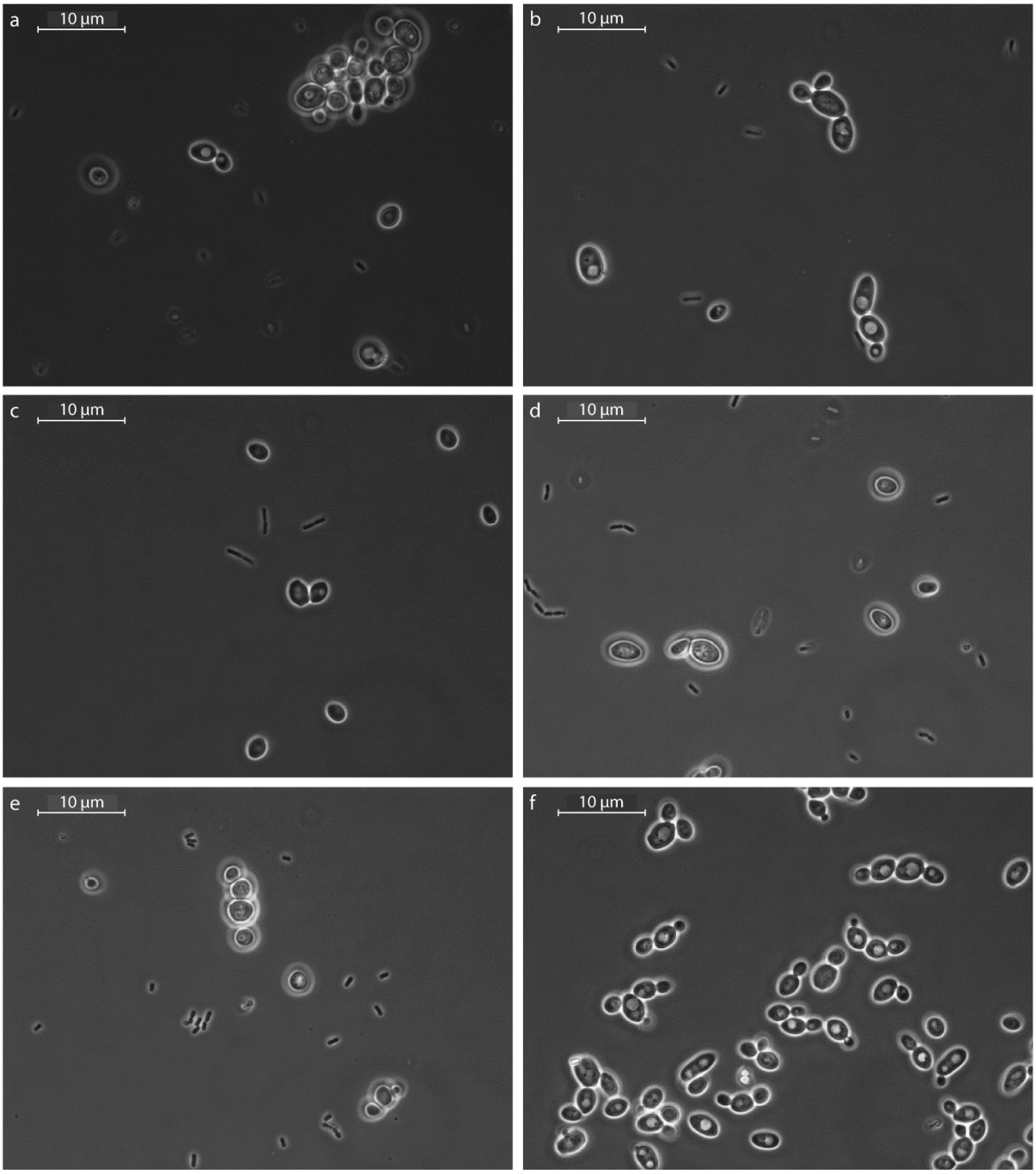

Figure S3. LAB-yeast pairwise co-cultures examined under light microscopy Images from selected isolate pairs after six transfers unless otherwise mentioned **a**, L.p2 and W.a2 **b**, L.p2 and P.fer **c**, L.p2 and K.mar **d**, L.p3 and P.kud **e**, L.p8 and T.del **f**, L.ros5 and P.fer, picture from 2nd transfer since only yeast (P.fer) was present.